Inappropriately low hepcidin levels in patients with myelodysplastic syndrome carrying a somatic mutation of *SF3B1*

Ilaria Ambaglio,[1] Luca Malcovati,[1,2] Elli Papaemmanuil,[3] Coby M. Laarakkers,[4,5] Matteo G. Della Porta,[1] Anna Gallì,[1,2] Matteo C. Da Vià,[1,2] Elisa Bono,[1,2] Marta Ubezio,[1,2] Erica Travaglino,[1] Riccardo Albertini,[6] Peter J. Campbell,[3] Dorine W. Swinkels,[4,5] and Mario Cazzola[1,2]

[1]Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [2]Department of Molecular Medicine, University of Pavia, Italy; [3]Cancer Genome Project, Wellcome Trust Sanger Institute, Hinxton, UK; [4]Department of Laboratory Medicine, Laboratory of Genetic, Endocrine and Metabolic Diseases, Radboud University Nijmegen Medical Center, The Netherlands; [5]Hepcidinanalysis.com, Nijmegen, The Netherlands; and [6]Department of Diagnostic Medicine, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

ABSTRACT

Somatic mutations of the RNA splicing machinery have been recently identified in myelodysplastic syndromes. In particular, a strong association has been found between *SF3B1* mutation and refractory anemia with ring sideroblasts, a condition characterized by ineffective erythropoiesis and parenchymal iron overload. We studied the relationship between *SF3B1* mutation, erythroid activity and hepcidin levels in myelodysplastic syndrome patients. Erythroid activity was evaluated through the proportion of marrow erythroblasts, soluble transferrin receptor and serum growth differentiation factor 15. Significant relationships were found between *SF3B1* mutation and marrow erythroblasts (P=0.001), soluble transferrin receptor (P=0.003) and serum growth differentiation factor 15 (P=0.033). Serum hepcidin varied considerably, and multivariable analysis showed that the hepcidin to ferritin ratio, a measure of adequacy of hepcidin levels relative to body iron stores, was inversely related to the *SF3B1* mutation (P=0.013). These observations suggest that patients with *SF3B1* mutation have inappropriately low hepcidin levels, which may explain their propensity to parenchymal iron loading.

Introduction

The majority of patients with myelodysplastic syndrome (MDS) present with anemia, and most of them become transfusion dependent during the clinical course of the disease.[1,2] Transfusion iron overload is, therefore, a common complication in MDS, although its impact on clinical outcome varies considerably in different MDS subtypes.[3]

The redistribution of transfusion iron from reticuloendothelial to parenchymal cells is modulated by hepcidin, a peptide that interacts with ferroportin inhibiting the release of iron from macrophages.[4] Hepcidin production is enhanced by iron and inflammation, suppressed by anemia and hypoxia, and negatively modulated by the erythroid marrow activity. In mouse models of beta thalassemia, ineffective erythropoiesis is typically associated with downregulation of hepcidin, upregulation of ferroportin in the duodenum, and increased iron absorption.[5] Interestingly, increased levels of growth differentiation factor 15 (GDF15) have been found in thalassemia, and GDF15 has been shown to inhibit hepcidin production in primary human hepatocytes.[6] Although the role of GDF15 as negative erythropoietic regulator of hepcidin is still debated, these observations suggest a potential link between ineffective erythropoiesis, release of bone morphogenetic proteins like GDF15, suppression of hepcidin production, and parenchymal iron loading in the so-called iron loading anemias.

Various pathogenetic mechanisms are responsible for anemia in MDS patients.[7] Ineffective erythropoiesis is mainly found in patients with low-risk disease, typically in refractory anemia with ring sideroblasts,[8] whereas reduced proliferation of the erythroid marrow is observed in those with advanced disease and excess of blasts.[7] Recently, we identified somatically acquired mutations in *SF3B1*, a gene encoding a core component of the RNA splicing machinery, in MDS patients with ring sideroblasts.[9,10] This genotype-phenotype correlation was also shown in subjects having a proportion of ring sideroblasts below the diagnostic threshold of 15%.[10] These observations strongly support a causal relationship between *SF3B1* mutations and ring sideroblasts,[9] and suggest a link between *SF3B1* mutations and both ineffective erythropoiesis and parenchymal iron loading.[8,10] To investigate this link, we studied the relationship between *SF3B1* mutation status, erythroid marrow activity, hepcidin level, and body iron status in a cohort of MDS patients.

Design and Methods

These investigations were approved by the Ethics Committee of the "Fondazione Istituto di Ricovero e Cura a Carattere Scientifico" (IRCCS) Policlinico San Matteo, Pavia, Northern Italy. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000, and samples were obtained after subjects provided informed consent.

We studied 76 patients with MDS or myelodysplastic/myeloproliferative neoplasm (MDS/MPN) followed at the Department of Hematology, "Fondazione IRCCS Policlinico San Matteo", Pavia,

 doi:10.3324/haematol.2012.077446
IA and LM contributed equally to this work. The online version of this article has a Supplementary Appendix.
Manuscript received on September 13, 2012. Manuscript accepted on December 28, 2012.
Correspondence: luca.malcovati@unipv.it

northern Italy. This cohort belongs to the patient population of our recent study on clinical significance of *SF3B1* mutations.[10] According to the 2001 and 2008 World Health Organization (WHO) classification criteria,[11,12] 26 patients had refractory anemia with ring sideroblasts (RARS) or refractory cytopenia with multilineage dysplasia (RCMD) and ring sideroblasts (RCMD-RS), 22 patients had refractory anemia (RA) with unilineage dysplasia or RCMD, 23 patients had refractory anemia with excess blasts (RAEB) type 1 (RAEB-1) or RAEB type 2 (RAEB-2), and 5 patients had RARS associated with marked thrombocytosis (RARS-T). Clinical and hematologic features of the patient cohort are reported in Table 1. Risk assessment was based on the WHO-based prognostic scoring system (WPSS)[13] accounting for severity of anemia.[3]

Soluble transferrin receptor (sTfR), a measure of total erythroid activity,[14] was quantified using an immunonephelometric method (Dade Behring Marburg GmbH, Marburg, Germany) on a BN II System analyzer. Serum erythropoietin (Epo) was assayed by a solid-phase chemiluminescent immunometric method (Immulite 2000 Analyzer, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The quantification of serum GDF15 was performed with DuoSet ELISA for human GDF15 (R&D Systems, Abingdon, UK). Serum ferritin was assayed using a nephelometric method (N Latex Ferritin, Siemens Healthcare Diagnostic Products, Marburg, Germany) on a BN II System analyzer.

Serum hepcidin-25 measurements were performed by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS).[15] Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionization TOF MS platform (Bruker Daltonics). Serum hepcidin-25 concentrations were expressed as nmol/L (nM). The lower limit of detection of this method was 0.5 nM; average coefficients of variation were 2.7% (intra-run) and 6.5% (inter-run). The reference range of serum hepcidin-25 is from less than 0.5 to 14.7 nM (median 4.5 nM) for men, from less than 0.5 to 12.3 nM (median 2.0 nM) for premenopausal women, and from less than 0.5 to 15.6 nM (median 4.9 nM) for postmenopausal women.

Mononuclear cells were separated from peripheral blood or bone marrow samples by standard density gradient centrifugation. Granulocyte and T-lymphocytes were isolated from peripheral blood as previously described.[16] Genomic DNA was extracted from granulocytes, T lymphocytes, or mononuclear bone marrow cells by following standard protocols for human tissue.

The coding exons of *SF3B1* were screened using massively parallel pyrosequencing of DNA pools using the genome sequencer FLX 454 system (Roche, Branford, CT, USA) as previously described in detail.[10]

Numerical variables are summarized by median and range; categorical variables are described with count and relative frequency (%) of subjects in each category. Comparison of numerical variables between groups was carried out using a non-parametric approach (Mann-Whitney test or Kruskall Wallis ANOVA). Correlations between quantitative variables were assessed using Spearman's rank correlation. Independent predictors of variables of interest were assessed using multivariable generalized linear regression models. Statistical analyses were performed using Stata 11.2 software (StataCorp LP, College Station, TX, USA).

Table 1. Demographic, clinical and hematologic features of the study population.

N. of patients	76
Male/female	40 (53%)/ 36 (47%)
Age (years)	67 (29-83)
WHO subgroups*	
RA/RCMD	22 (29%)
RARS/RCMD-RS	26 (34%)
RAEB-1/RAEB-2	23 (30%)
RARS-T	5 (7%)
WPSS risk group†	
Very low	11 (15%)
Low	26 (37%)
Intermediate	12 (17%)
High	18 (25%)
Very high	4 (6%)
Karyotype	
Normal	34 (45%)
Abnormal	38 (50%)
Not available	4 (5%)
Transfusion dependency	25 (33%)
Complete blood count	
Hemoglobin (g/dL), median (range)	9.4 (6.1-13.7)
Absolute neutrophil count (x10⁹/L), median (range)	2.2 (1.9-12.6)
Platelet count (x10⁹/L), median (range)	134 (17-797)
Bone marrow features	
Bone marrow blasts (%), median (range)	3 (0-19)
Bone marrow erythroblasts (%), median (range)	26 (8-79)
Bone marrow ring sideroblasts(%), median (range)	8 (0-94)
Dyserythropoiesis:	
Mild	15 (20%)
Moderate	38 (50%)
Severe	23 (30%)
Serum iron (g/dL), median (range)	167 (61-346)
Transferrin (mg/dL), median (range)	184 (94-329)
Transferrin saturation (%), median (range)	53 (20-100)
Serum ferritin (ng/mL), median (range)	409 (6-8340)

WHO: World Health Organization; RA: refractory anemia; RCMD: refractory cytopenia with multilineage dysplasia; RARS: refractory anemia with ring sideroblasts; RCMD-RS: refractory cytopenia with multilineage dysplasia and ring sideroblasts; RAEB-1: refractory anemia with excess blasts type 1; RAEB-2: refractory anemia with excess blasts type 2; RARS-T: refractory anemia with ring sideroblasts associated with marked thrombocytosis. WPSS: WHO classification-based Prognostic Scoring System. WPSS risk was not estimated in the 5 RARS-T patients.

Results and Discussion

Somatic mutations of *SF3B1* were found in 21 of 76 (28%) patients. The proportion of positive patients was significantly higher in WHO categories defined by ring sideroblasts (17 of 31 or 55%) than in other categories (4 of 45 or 9%) (P<0.001). The median value for *SF3B1* mutant allele burden was 45% (range 7%-64%).

The relationship between *SF3B1* mutant allele burden and erythroid activity is illustrated in Figure 1, while additional information is provided in the *Online Supplementary Appendix*. There was a significant relationship between *SF3B1* mutation and sTfR level (P=0.003), and between *SF3B1* mutant allele burden and sTfR (r=0.38, P=0.001). A significant association between *SF3B1* mutation and GDF15 levels was observed, patients carrying a *SF3B1* mutation having higher values than those without mutation (P=0.033). In addition, a positive correlation was found between *SF3B1* mutant allele burden and GDF15 (r=0.28, P=0.016).

Variable hepcidin levels were found in the patients studied (median value 7.1 nM, range from less than 0.5 to 92.0 nM). There was a significant relationship between hepcidin and serum ferritin (r=0.59, P<0.001). A significant interac-

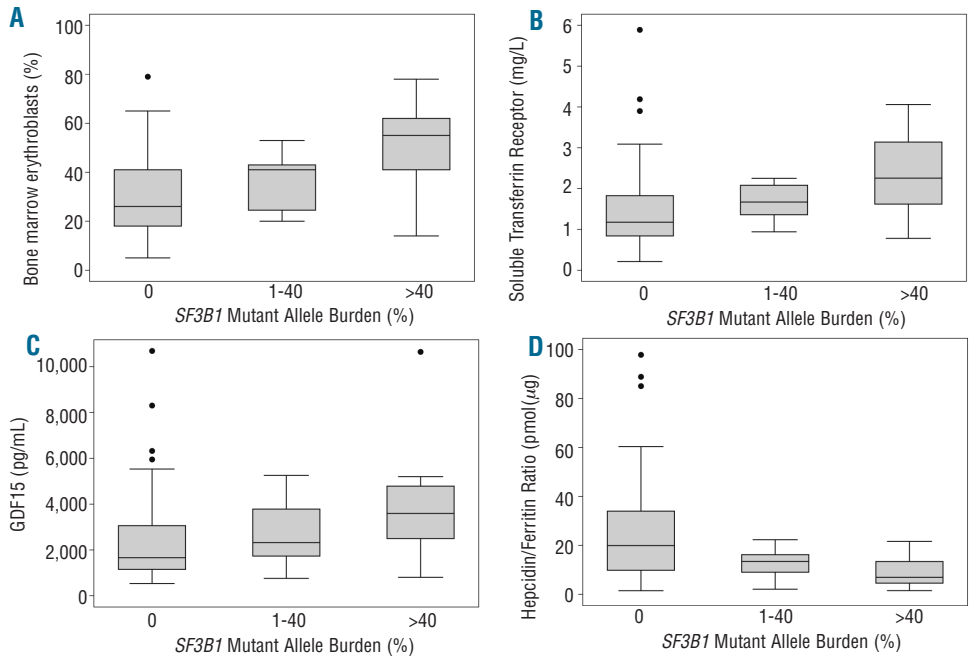

Figure 1. Relationship between *SF3B1* mutant allele burden and measurements of erythroid marrow activity or hepcidin to ferritin ratio in MDS patients. (A) Relationship between *SF3B1* mutant allele burden and bone marrow erythroblasts (r=0.46, P<0.001). (B) Relationship between SF3B1 mutant allele burden and soluble transferrin receptor level, a measure of total erythroid activity (r=0.38, P=0.001). (C) Relationship between *SF3B1* mutant allele burden and serum GDF15 concentration (expressed as ng/mL), a measure of ineffective erythropoiesis (r=0.28, P=0.016). (D) Relationship between hepcidin to ferritin ratio and *SF3B1* mutant allele burden. *SF3B1* mutant allele burden was categorized into groups of comparable size (0, 1-40%, and >40%). Data are shown in a box plot depicting the upper and lower adjacent values (lowest and highest horizontal line, respectively), lower and upper quartile with median value (box), and outside values (dots).

tion between *SF3B1* mutation and serum ferritin was observed in a multivariable linear regression model on serum hepcidin level (P=0.005), suggesting a blunted hepcidin response to iron overload in *SF3B1*-mutated patients (reflected in the lower slope of the gray regression line in Figure 2).

We then calculated the hepcidin to ferritin ratio, which represents a measure of adequacy of hepcidin levels relative to body iron stores. Significantly lower values of hepcidin to ferritin ratio were found in patients with *SF3B1* mutation compared with those without mutation (P=0.004), and a significant relationship was observed between hepcidin to ferritin ratio and *SF3B1* mutant allele burden (r=-0.37, P=0.001) (Figure 1). The relationship between hepcidin to ferritin ratio and measurements of erythroid activity is illustrated in *Online Supplementary Figure S1*.

Multivariable analyses were performed in order to identify the most important factors affecting hepcidin level and hepcidin to ferritin ratio in MDS patients (*Online Supplementary Table S1*). In a multivariable analysis including hemoglobin, Epo, sTfR, GDF15, bone marrow blasts, cytogenetic risk groups and *SF3B1* mutant allele burden, the hepcidin to ferritin ratio was independently associated with *SF3B1* mutant allele burden (P=0.013).

The above findings indicate that MDS patients carrying a somatic mutation of *SF3B1* not only have a distinct clinical phenotype characterized by ring sideroblasts,[9,10] but show also evidence of ineffective erythropoiesis and relative hepcidin deficiency, i.e. typical features of iron loading anemias. In particular, patients with *SF3B1* mutation had high proportions of bone marrow erythroblasts and high levels of sTfR, both measures of total erythroid activity, and slightly increased levels of GDF15, a measure of ineffective erythropoiesis.[6,17-19]

The *SF3B1* mutant allele burden was independently associated with hepcidin levels and the hepcidin to ferritin ratio.

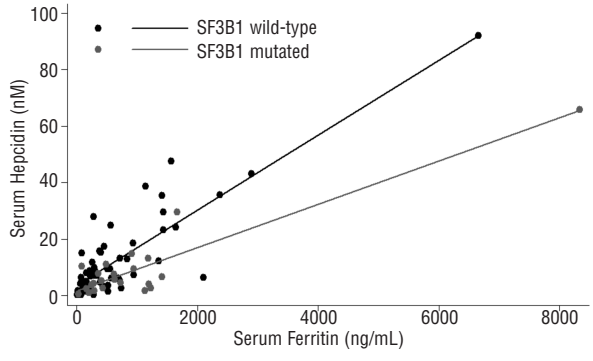

Figure 2. Linear dependence between hepcidin and serum ferritin according to *SF3B1* mutation status in patients with MDS. Dots and regression lines of hepcidin and serum ferritin are drawn in black and gray for patients without and with *SF3B1* mutation, respectively. The significant interaction (P=0.005) between *SF3B1* mutation and serum ferritin obtained in a multivariable linear model for serum hepcidin level is reflected in the lower slope of the gray regression line, indicating a blunted hepcidin response to iron overload in *SF3B1*-mutated patients.

This suggests that expanded but ineffective erythropoiesis may suppress hepcidin production in MDS patients carrying a mutation of *SF3B1*, and lead to hepcidin levels that are inappropriately low relative to body iron stores.[20] Consistent with this observation, in a previous study on hepcidin levels in MDS,[21] WHO category remained a significant predictor of hepcidin in multivariate analyses adjusted for ferritin and transfusion history, and the lowest levels were indeed found in RARS patients.

It should be noted, however, that in our study the *SF3B1* mutation retained an effect on hepcidin level independently of erythroid marrow activity, suggesting that the mutant

splicing factor may contribute to hepcidin suppression through mechanisms other than ineffective erythropoiesis. Recent studies showed that *SF3B1* mutation is associated with downregulation of essential mitochondrial gene networks[9] and more specifically of *ABCB7*,[22] and that *SF3B1* haploinsufficiency leads to formation of ring sideroblasts.[23] It has been previously shown that iron sequestration into mitochondria results in cytosolic iron depletion and increased cellular iron uptake.[24,25] Therefore, the possibility exists that abnormal mitochondrial iron homeostasis has an effect on hepcidin production.

In conclusion, our study suggests that MDS patients carrying a somatic mutation of *SF3B1* have inappropriately low hepcidin levels which may cause excessive reticuloendothelial iron release and parenchymal iron loading. This may be relevant for decision-making concerning treatment of transfusion iron overload in MDS patients.[26] Taking into account life expectancy,[2,13] clinical consequences of transfusion iron overload,[3] and adequacy of hepcidin production, transfusion-dependent patients with refractory anemia with ring sideroblasts appear to be those more likely to develop deleterious consequences of parenchymal iron overload. These patients may, therefore, benefit from iron chelation therapy, a very controversial issue in the MDS community. Despite considerable skepticism, recent studies have shown improvements in hematologic and hepatic parameters in MDS patients receiving iron chelation.[27,28]

Funding

This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC, "Special Program Molecular Clinical Oncology 5x1000", project n.1005) and Fondazione Cariplo to MC, and from Fondazione Berlucchi per la Ricerca Oncologica to MGDP and LM.

Acknowledgments

The authors wish to thank Dr. Cristiana Pascutto for her invaluable help in performing statistical analyses.

Authorship and Disclosures

Information on authorship, contributions, and financial and other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Cazzola M, Malcovati L. Myelodysplastic syndromes--coping with ineffective hematopoiesis. N Engl J Med. 2005; 352(6): 536-8.
2. Malcovati L, Della Porta MG, Pascutto C, Invernizzi R, Boni M, Travaglino E, et al. Prognostic factors and life expectancy in myelodysplastic syndromes classified according to WHO criteria: a basis for clinical decision making. J Clin Oncol. 2005;23 (30):7594-603.
3. Malcovati L, Della Porta MG, Strupp C, Ambaglio I, Kuendgen A, Nachtkamp K, et al. Impact of the degree of anemia on the outcome of patients with myelodysplastic syndrome and its integration into the WHO classification-based Prognostic Scoring System (WPSS). Haematologica. 2011;96 (10):1433-40.
4. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004;306(5704):2090-3.
5. Gardenghi S, Marongiu MF, Ramos P, Guy E, Breda L, Chadburn A, et al. Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood. 2007;109(11): 5027-35.
6. Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med. 2007;13(9):1096-101.
7. Cazzola M, Barosi G, Berzuini C, Dacco M, Orlandi E, Stefanelli M, et al. Quantitative evaluation of erythropoietic activity in dysmyelopoietic syndromes. Br J Haematol. 1982;50(1):55-62.
8. Cazzola M, Barosi G, Gobbi PG, Invernizzi R, Riccardi A, Ascari E. Natural history of idiopathic refractory sideroblastic anemia. Blood. 1988;71(2):305-12.
9. Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. N Engl J Med. 2011;365(15): 1384-95.
10. Malcovati L, Papaemmanuil E, Bowen DT, Boultwood J, Della Porta MG, Pascutto C, et al. Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. Blood. 2011;118(24):6239-46.
11. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100(7):2292-302.
12. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008.
13. Malcovati L, Germing U, Kuendgen A, Della Porta MG, Pascutto C, Invernizzi R, et al. Time-dependent prognostic scoring system for predicting survival and leukemic evolution in myelodysplastic syndromes. J Clin Oncol. 2007;25(23):3503-10.
14. Cazzola M, Beguin Y, Bergamaschi G, Guarnone R, Cerani P, Barella S, et al. Soluble transferrin receptor as a potential determinant of iron loading in congenital anaemias due to ineffective erythropoiesis. Br J Haematol. 1999;106(3):752-5.
15. Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede AE, et al. Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clinical Chemistry. 2010;56(10):1570-9.
16. Malcovati L, Della Porta MG, Pietra D, Boveri E, Pellagatti A, Galli A, et al. Molecular and clinical features of refractory anemia with ringed sideroblasts associated with marked thrombocytosis. Blood. 2009; 114(17):3538-45.
17. Tamary H, Shalev H, Perez-Avraham G, Zoldan M, Levi I, Swinkels DW, et al. Elevated growth differentiation factor 15 expression in patients with congenital dyserythropoietic anemia type I. Blood. 2008;112(13):5241-4.
18. Ramirez JM, Schaad O, Durual S, Cossali D, Docquier M, Beris P, et al. Growth differentiation factor 15 production is necessary for normal erythroid differentiation and is increased in refractory anaemia with ringsideroblasts. Br J Haematol. 2009;144(2): 251-62.
19. Casanovas G, Swinkels DW, Altamura S, Schwarz K, Laarakkers CM, Gross HJ, et al. Growth differentiation factor 15 in patients with congenital dyserythropoietic anaemia (CDA) type II. J Mol Med. 2011; 89(8):811-6.
20. Papanikolaou G, Tzilianos M, Christakis JI, Bogdanos D, Tsimirika K, MacFarlane J, et al. Hepcidin in iron overload disorders. Blood. 2005;105(10):4103-5.
21. Santini V, Girelli D, Sanna A, Martinelli N, Duca L, Campostrini N, et al. Hepcidin levels and their determinants in different types of myelodysplastic syndromes. PLoS One. 2011;6(8):e23109.
22. Nikpour M, Scharenberg C, Liu A, Conte S, Karimi M, Mortera-Blanco T, et al. The transporter ABCB7 is a mediator of the phenotype of acquired refractory anemia with ring sideroblasts. Leukemia 2012 Oct 16. doi: 10.1038/leu.2012.298. [Epub ahead of print].
23. Visconte V, Rogers HJ, Singh J, Barnard J, Bupathi M, Traina F, et al. SF3B1 haploinsufficiency leads to formation of ring sideroblasts in myelodysplastic syndromes. Blood. 2012;120(16):3173-86.
24. Nie G, Sheftel AD, Kim SF, Ponka P. Overexpression of mitochondrial ferritin causes cytosolic iron depletion and changes cellular iron homeostasis. Blood. 2005;105 (5):2161-7.
25. Della Porta MG, Malcovati L, Invernizzi R, Travaglino E, Pascutto C, Maffioli M, et al. Flow cytometry evaluation of erythroid dysplasia in patients with myelodysplastic syndrome. Leukemia. 2006;20(4):549-55.
26. Ghoti H, Fibach E, Westerman M, Gordana O, Ganz T, Rachmilewitz EA. Increased serum hepcidin levels during treatment with deferasirox in iron-overloaded patients with myelodysplastic syndrome. Br J Haematol. 2011;153(1):118-20.
27. List AF, Baer MR, Steensma DP, Raza A, Esposito J, Martinez-Lopez N, et al. Deferasirox reduces serum ferritin and labile plasma iron in RBC transfusion-dependent patients with myelodysplastic syndrome. J Clin Oncol. 2012;30(17):2134-9.
28. Gattermann N, Finelli C, Della Porta M, Fenaux P, Stadler M, Guerci-Bresler A, et al. Hematologic responses with deferasirox therapy in transfusion-dependent myelodysplastic syndromes patients. Haematologica. 2012;97(9):1364-71.